UNITED STATES PATENT APPLICATION

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FOR: APPARATUS FOR MEASURING THE VOLUME
OF INDIVIDUAL RED BLOOD CELLS

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FIELD OF THE INVENTION

The present invention relates to the field of quantitative microspectroscopy, and in

10   particular to an apparatus and a method for measuring the volume of individual red blood

cells.

BACKGROUND OF THE INVENTION

Determining the volume of individual red blood cells and, based on this measurement,

15   calculating the Mean Cell Volume ("MCV") and the Red Cell Distribution Width ("RDW")

is of clinical interest. Usually, systems based on electrical impedance measurement (Coulter

Counter) or based on light scattering (Flow Cytometer) are employed (see. e.g., J.B. Henry,

"Clinical diagnosis and management by laboratory methods", W.B. Saunders Company,

Philadelphia, 1996, pp. 548 ff. or D.H. Tycko, M.H. Metz, E.A. Epstein, A. Grinbaum,

20   "Flow-cytometric light scattering measurement of red blood cell volume and hemoglobin

concentration", Applied Optics 24 (1985), 1355 – 1365). Impedance counters are complex

and expensive instruments that require very careful adjustment and control of instrument and

sample parameters. A major disadvantage of flow cytometers is the fact that the parameters

of light scattering depend not only on cell volume, but also on the cell's shape.

In 1983, Gray, Hoffman and Hansen proposed a new optical method for determining

the volume of cells in a flow cytometer (M.L. Gray, R.A. Hoffman, W.P. Hansen, "A new

method for cell volume measurement based on volume exclusion of a fluorescent dye",

5   Cytometry 3 (1983), 428 – 432). In this method, the cells are suspended in a fluorescent dye,

which is unable to penetrate the cell membrane. The level of fluorescence, which is

produced when a narrow stream of the cell suspension is excited by a focused laser beam,

will remain constant until a cell arrives in the illuminated region thereby causing a decrease

in fluorescence intensity, which is directly proportional to the volume of the cell. In a flow

10   cytometer, a single cell is passing through the laser-illuminated spot within approximately

10. Due to this short data acquisition time interval, the electronic detection bandwidth has

to be relatively large, which results in a poor signal-to-noise ratio and in a low precision for

the volume determination.

The available data acquisition time can be significantly increased by suspending the

15   cells in a stationary sample and applying digital imaging fluorescence microscopy (see P.L.

Becker, F.S. Fay, "Cell-volume measurement using the digital imaging fluorescence

microscope", Biophysical Journal 49 (1986), A465). In the digital fluorescence microscopy

approach, a calibration procedure is required in order to determine the cell volume.

Recktenwald and co-workers have introduced a method where the calibration is performed

20   by means of optical transparent and non-fluorescent microspheres that are suspended

together with the cells (D. Recktenwald, J. Phi-Wilson, B. Verwer, "Fluorescence quantitation using digital microscopy", Journal Physical Chemistry 97 (1993), 2868 – 2870). The volume of individual spheres is determined by measuring their projection area under the microscope and transforming this number into a volume, assuming an ideal spherical shape.

5   The decrease in fluorescence intensity as a result of the spheres' volume that is being excluded from emitting fluorescence is used as the required calibration parameter. The advantage of this approach is given by the fact that the calibrating particles are located within the sample itself. In other words, a calibration is performed on the very same sample container, and no extra calibration sample is required.

10   The use of calibration spheres within a cell suspension is not without problems. First, the introduction of the spheres represents an additional step in the workflow. In systems that are designed for high throughput, this additional step would represent a disadvantage. Secondly, Recktenwald and co-workers observed a tendency of the fluorescent dye molecules to settle down on the sphere's surface, which causes an error. Third, if the optical

15   index of refraction of the spheres does not match well with the liquid's index, then refraction-based artifacts in the measured fluorescence intensity occur at the edges of the spheres. And, finally, the use of microspheres can represent a problem, if, for example, a thin sample thickness in the order of a few micrometers or less is needed.

In view of the disadvantages and problems in the prior art as described above, there

20   exists a need for a simple and reliable method for measuring the volume of individual red

blood cells suspended in a liquid.

SUMMARY OF THE INVENTION

It is an objective of the present invention to provide a method and apparatus for measuring the volume of individual red blood cells or other particles that are suspended in

5   liquids.

According to the present invention, and for the case of red blood cells, the above objective is achieved by depositing a liquid sample that contains suspended red blood cells into an optical cuvette having an input window and an output window, by adding and evenly distributing an absorbing dye into the liquid that does not leak into the red blood cells, and

10   that is able to absorb light at wavelengths that are only weakly absorbed by the red blood cells, by illuminating the sample through the input window at a wavelength that is absorbed by said dye, but only weakly absorbed by the red blood cells, by measuring the transmitted light intensity reemerging through the output window in an area that contains no red blood cells, by changing the cuvette thickness in that area by a well-defined amount and measuring

15   the reemerging light intensity in the same area again, by measuring the reemerging light intensity in an area where a red blood cell resides, by measuring the reemerging light intensity in an area close to that same red blood cell, and by calculating the volume of the red blood cell based on these light intensity values and the known change in cuvette thickness.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 depicts a measuring set-up with an optical cuvette having a stiff input window

5 and a flexible output window, and containing a liquid sample comprising suspended red blood cells.

FIG. 2 illustrates a complete set-up comprising sample container, XYZ-stage, transmission microscope, CCD camera, and computer.

FIG. 3 shows schematically an optical cuvette with a region containing a weakly

10 absorbing red blood cell, and another region containing no cells. The arrows illustrate the stream of photons traversing the sample, whereby the decrease in width is meant as an indication of the decreasing light intensity as a result of absorption.

FIG. 4 illustrates the calibration procedure, where the cuvette thickness in an area that contains no red blood cells is changed by a well-defined amount, h, and the reemerging light

15 intensities, I_1 and I_2, are measured prior to and after changing the thickness. In this example, the cuvette thickness has been reduced.

FIG. 5 illustrates the measuring procedure, where the reemerging light intensities, I_4 and I_3, are measured in an area where a red blood cell resides, and in an area close to that same cell. In FIG. 5, we assume a cylindrical cell of height, h_{RBC}.

20 FIG. 6 shows a calculated plot of reemerging light intensity in arbitrary units, if a

cuvette thickness of approximately 18 m is increased in ten steps of 0.2 m each, up to

approximately 20 m. In this example, TO-PRO-3 in a concentration c = 1 mM/L has been

assumed as the absorbing dye, which has an absorption coefficient a = 1.144 * 10^5 L/M cm

at 640 nm wavelength.

5   ## DETAILED DESCRIPTION OF THE INVENTION

According to the method and apparatus of the present invention, a liquid sample that

contains suspended red blood cells is deposited into an optical cuvette having an input

window and an output window. Preferably, the cuvette is relatively thin and suitable to be

positioned onto the sample stage of a transmission microscope. For example, the cuvette can

10   be built by placing a flexible #1 coverslip of 24 mm x 50 mm size onto spacers that are

residing on a common microscope slide of 25 mm x 75 mm size. The preferred height of the

spacers is approximately 200 m. By pressing onto the flexible coverslip, thickness values

in the center region of the cuvette between less than 1 m and 200 m can be achieved. An

optical cuvette is any container that is able to hold a liquid sample within its interior space

15   and that has a transparent entrance window and a transparent exit window, usually on

opposite sides, allowing for transmission measurement. It would also be within the spirit of

the invention to use a container with one transparent window on one side and a mirror on the

other side. In this case, light would enter the container through said one transparent window,

would cross the liquid sample twice, and would exit the container through the same window.

20   The one window would function as both entrance window and exit window. The invention

is not limited to containers for microscopic analysis, but is applicable also for containers of larger size that are interrogated on optical systems other than microscopes.

An absorbing dye is added to, and evenly distributed within the liquid sample. The dye is selected so that it does not leak into the red blood cells. Also, it should absorb

5   excitation light within a spectral region where the absorption within the red blood cells is only weak. Since hemoglobin is the dominant absorber in red blood cells, the illumination wavelength has to be longer than 600 nm. One good candidate dye is TO-PRO-3 (sold, for example, by Molecular Probes, Inc., Eugene, Oregon), that can be illuminated within a wavelength range around to 640 nm, where it has an absorption coefficient of $1.14*10^5$

10   L/Mcm. Another possible dye would be TO-PRO-5 (sold by Molecular Probes, Inc.), which also does not penetrate into the red blood cells, can be illuminated around 750 nm, and has an absorption coefficient of $1.21*10^5$ L/Mcm.

It is apparent to one skilled in the art that the invention is not limited to the two dyes mentioned above. Many other dyes are available that fulfill the spectral conditions for

15   measurements on red blood cells, and even more dyes are available that fulfill the spectral conditions for other particles. It would of course also still be within the spirit of the present invention to add and evenly distribute the absorbing dye within the liquid sample prior to disposing the sample into the container.

FIG. 1 depicts a measuring set-up with an optical cuvette (20) having a stiff input

20   window (1), a flexible output window (3) and containing a liquid sample (4) comprising

suspended red blood cells. The cuvette is built by using a common microscope slide (1) that

carries spacers (2, 2') to hold a flexible cover slip (3). The suspension of red blood cells in

a liquid such as blood plasma (4) is contained between slide (1) and cover slip (3). The

optical cuvette is positioned on an XYZ-stage (5) of a common transmission microscope (7)

5   having interchangeable objective lenses (8, 9, and 10) and a sample illumination source (6).

As shown in FIG.2, microscope (7) is equipped with a CCD camera (21) that is connected

to a computer (22) for storing data and performing image-processing procedures. Computer

(22) is also connected to XYZ-stage (5) to move cuvette (20) as needed.

FIG. 3 shows schematically an optical cuvette with a region containing a weakly

10   absorbing red blood cell, and another region containing no cells. The arrows illustrate the

stream of photons traversing the sample, whereby the decrease in width is meant as an

indication of the decreasing light intensity as a result of absorption. As indicated in FIG. 3,

the optical absorption within a red blood cell is so weak that no decrease in intensity occurs.

In operation, and referring again to FIGS. 1 and 2, cuvette (20) is moved upwards by

15   means of stage (5) until flexible window (3) comes into physical contact with a fixed plunger

(11) that is mounted onto objective lens (10) in such a way that the focal plane of microscope

(7) lies within liquid sample (4) if flexible window (3) is touching plunger (11). If necessary,

cuvette (20) is being moved in X- and Y-directions until a sample area containing no red

blood cells comes into the field of view. If the sample is whole blood, then it is appropriate

20   to use a cuvette thickness in the range of 2 m to 30 m in order to have areas containing no

red blood cells readily available. For diluted blood samples, thicker cuvettes can be used.

Once an area containing no red blood cells has been found, the sample is illuminated with light of appropriate wavelength by means of illumination source (6), and the reemerging intensity of the transmitted light, I_1, in that area is measured by means of CCD camera (21),

5 and the result stored in computer (22). This is illustrated in FIG. 4. In a next step, cuvette (20) is moved further upwards by means of stage (5) by a small, but precisely known distance, h. Moving cuvette (20) upward against fixed plunger (11) results in a reduction in the optical path length within the cuvette by an amount identical to h. When cuvette (20) has been moved, a new reemerging light intensity, I_2, in the same area is measured, and the result

10 stored in computer (22). The new light intensity, I_2, has a higher value than the first intensity, I_1, because a smaller sample thickness results in less absorption of light within the sample.

The two reemerging light intensity values, I_1 and I_2, together with the change in optical path length, h, can be used to calibrate the set-up by calculating a ratio "change in

15 reemerging light intensity/change in sample thickness". Referring to FIG. 4, this calibration can be explained as follows.

Assuming that the liquid sample is evenly illuminated by excitation light, the first reemerging light intensity, I_1, is given by

$$(1) \qquad I_1 = I_0 * 10^{-ac*h_1}$$

where I_0 is the illumination intensity, a is the absorption coefficient of the light-absorbing dye, c is the concentration of the dye. The quantity h_1 is the optical path length (or thickness) of cuvette (20) at the area that is measured.

The light intensity, I_2, which is measured after changing the cuvette thickness by an

5   amount of h is given by

$$(2) \qquad I_2 = I_0 * 10^{-ac*(h_1 - \Delta h)} \ .$$

By dividing equation (2) by equation (1), and by taking the logarithm, we obtain an expression for the product a*c, which represents our calibration quantity:

$$(3) \qquad a*c = \frac{lg(\frac{I_2}{I_1})}{\Delta h} \ .$$

10   Equation (3) indicates that there is no need for knowing the absolute cuvette thickness, h_1, or for measuring the illumination intensity, I_0, because these two quantities are canceled out.

After performing a calibration as outlined above, in the method according to the present invention, the volume of a single red blood cell is determined in the following way:

In a first step, the reemerging light intensity, I_4, is measured in an area, A_{RBC}, where a red blood cell resides. For the moment, we assume that the red blood cell has a cylindrical

5   shape of area A_{RBC}, and of height h_{RBC} (see FIG. 5). We also assume a height of the liquid sample layer, h_2, which has not to be equal to h_1, and an illumination intensity, I'_0, which has not to be identical to I_0. Under these conditions, we obtain

$$(4) \qquad I_4 = I'_0 * 10^{-a\,c*(h_2 - h_{RBC})}$$

for the reemerging light intensity.

10   In a second step, the reemerging light intensity, I_3, is measured in an area of identical size, A_{RBC}, close to that same red blood cell. Under the same conditions as for I_4, we obtain

$$(5) \qquad I_3 = I'_0 * 10^{-a\,c*h_2} \; .$$

Dividing equation (4) by equation (5), we obtain

or

$$(6) \quad lg(\frac{I_4}{I_3}) = a\,c * h_{RBC}$$

$$(7) \quad h_{RBC} = \frac{lg(\frac{I_4}{I_3})}{a * c} \; .$$

For a cylindrical cell of area A_{RBC}, the cell volume, V_{RBC}, can be calculated by multiplying the cell area by the cell height as given in equation (7), which results in

$$(8) \quad V_{RBC} = A_{RBC} * h_{RBC} = A_{RBC} * \frac{lg(\frac{I_4}{I_3})}{a * c} \; ,$$

5   or, with equation (3) for the product a*c, in

$$(9) \quad V_{RBC} = A_{RBC} * \Delta h * \frac{lg(\frac{I_4}{I_3})}{lg(\frac{I_2}{I_1})}.$$

Equation (9) shows that the method according to the present invention is based on differential measurements regarding the thickness of the cuvette, and on ratiometric measurements regarding the reemerging light intensities. In other words, there is no need to know the absolute thickness of the cuvette. Moreover, there is no need to know the excitation intensity which is injected into the cuvette.

Equation (9) shows also that any long-term drift in the instrumental parameters is canceled out. This results from the fact that the volume of a red blood cell, V_{RBC}, is calculated from a ratio of photocurrents. In other words, if the calibration procedure is executed closely in time with the measurement, then the method according to the present invention is very robust. This condition will always be fulfilled, since the calibration procedure is performed on the very same sample that is being measured.

So far, it has been assumed that the red blood cell has a cylindrical shape. It can be shown that the shape of the red blood cell can be irregular and that the Z - position of the cell within the cuvette has no impact on the calculated cell volume. The volume of a cell, V_{RBC}, is related to the spatially dependent cell height, h_{RBC}, via the equation

$$(10) \quad V_{RBC} = \int h_{RBC}(\xi,\eta)\, d\xi\, d\eta \ .$$

In equation (10), the quantities and represent the independent X- and Y-variables within the

red blood cell. The required integration is easily performed by adding the pixel intensities,

$I_4(\ ,\ )$, within the area, A^*_{RBC}, that is being occupied by the red blood cell on the photosensitive

$$(11) \quad V_{RBC} = (1/ac) * \int lg\left(\frac{I_4(\xi,\eta)}{I_3}\right) d\xi\, d\eta \ ,$$

5   target of the CCD camera. Combining equations (7) and (10) results in

which represents the final expression for the red blood cells volume as determined according

to the present invention.

The integration procedure shown in equation (11) has not to be performed exactly

over the area occupied by a red blood cell. Instead, it could be performed over a somewhat

10   larger area that includes the cell. Outside of the cell, $I_4(\xi,\eta)$ is identical to I_3; therefore

$lg[I_4(\xi,\eta)/I_3] = 0$. In other words, extending the integration over a larger area beyond the

cell's perimeter would not result in additional contributions to the calculated cell volume.

This aspect of the invention allows for the use of simple one-size one-shape integration areas

for all individual cells that are studied. Consequently, the required calculations can be

executed within a shorter time interval. It should be noted that it is assumed, as equation (11)

implies, that the intensity I_3 near the cell is constant. While $I_4(\xi,\eta)$ is measured in practice

as an intensity of single CCD pixels, I_3 can be determined with maximum precision as the

sum of all pixel intensities over an area, divided by the number of pixels. In other words, I_3

5   is the average pixel intensity near the cell.

In order to enhance the calibration precision, it is possible to employ more than one

change, h, in cuvette thickness. FIG. 6 shows a calculated plot of reemerging light intensity in

arbitrary units, if a cuvette thickness of approximately 18 m is increased in ten steps of 0.2 m

each, up to approximately 20 m. In this example, TO-PRO-3 in a concentration c = 1 mM/L

10   has been assumed as the absorbing dye, which has an absorption coefficient a = $1.144 * 10^5$

L/M cm at 640 nm wavelength. As can be seen from FIG. 6, within the relatively small range

of change in thickness, the light intensity as a function of thickness can be represented by a

straight-line relationship. Using the full 5.4-% change in intensity shown by the dashed line in

FIG. 6, which represents the best fit, we calculate a value of a*c = $lg(0.6223/0.5903)/(2.0*10^{-4}$

15   cm) = 114.63 cm^{-1}, which, of course, matches well with the expected value of 114.44 cm^{-1}.

The method for measuring the volume of individual red blood cells according to the

present invention can be applied to whole blood or to diluted blood samples. The present

invention can also be applied to a whole cluster of red blood cells or other particles in a

liquid suspension, and not only to individual particles or cells. It would also be within the

20   spirit of the invention to apply this method to other particles suspended in liquids such as

beads or other particles or cells, for example, prokaryotic, bacterial, eukaryotic, mammalian, tissue culture or human cells. It is also apparent to one skilled in the art that the present invention can also be applied to particles or cells in other liquid suspensions or samples or dilutions thereof, such other medical or biological samples including tissue cultures or other

5   cells in culture, including bacterial cultures, and other body fluids such as blood, urine, sputum, saliva and lymph fluid.

Depending on the optical properties of a liquid sample, the method can also be applied in cuvettes of higher thickness. Using a flexible window is only one example for achieving a change in the thickness of the cuvette. It would also be possible to use a stiff

10   window, but spacers made out of a flexible material such as rubber. Still another embodiment would be possible by utilizing a flexible cuvette wall instead of localized spacers. Also, the change in cuvette thickness can be achieved by leaving the main part of the cuvette fixed, and acting with a positive or negative force onto the window, so that the window is moving. Such positive or negative forces may even involve the use of pressure

15   or vacuum.

As has been already mentioned above, the invention is not limited to optical microscopes. Any imaging system that allows measuring transmitted light intensities in areas that contain a particle and in areas that do not contain a particle are suitable to practice the present invention. The imaging system may contain lenses, but may also use fiber-optic

20   elements in so-called proximity configurations. In this case, a coherent fiber-optic bundle

is arranged between a cuvette window and an imaging photodetector. Finally, it would still be within the spirit of the present invention to scan a beam of light across the cuvette windows and to monitor the transmitted light with a non-imaging photodetector, while the thickness of the cuvette is changing.

5   It should be mentioned that the step of calibration, i.e. determining the quantity "change in reemerging light intensity / change in sample thickness" according to equation (3) can also be performed in close proximity to the particle under investigation. In this case, and referring to Fig. 4, one would measure first the reemerging light intensity in the neighborhood of a single particle. In a next step, the thickness of the optical cuvette in this

10   area is being changed by an amount Δh, and new reemerging light intensity is measured. In a third step, the reemerging light intensity in the area occupied by the particle is measured. While the first two steps provide the required calibration, the second and the third step provide the two reemerging light intensity values that are needed for the actual volume measurement. This second procedure according to the present invention has the advantage

15   that three instead of four steps are required. The first procedure according to the present invention allows performing the calibration within a cuvette area of increased thickness, which would allow for a more precise calibration value due to the increased reemerging light intensity levels. In practice, the user has to decide, based on the priorities at hand.

          While the present invention is described herein in terms of certain preferred

20   embodiments, those skilled in the art will recognize that various modifications and

improvements may be made to the invention without departing from the scope thereof.

Moreover, although individual features of one embodiment are not in other embodiments,

it would be apparent that individual features of one embodiment may be combined with one

or more features of another embodiment or features from a plurality of embodiments.